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Further report of the occurrence of tetrodotoxin and new analogues in the Anuran family Brachycephalidae

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Abstract

Tetrodotoxin (TTX) is one of the most potent toxin already isolated, which occurs in a wide range of marine as well as terrestrial animals such as in newts and anurans. In this work, the occurrence of TTX and analogues was examined in three brachycephalid species: *Brachycephalus ephippium*, *B. nodoterga* and *B. pernix* using LC-FLD and LC-MS/MS. In toxicity assay (intra-peritonial injection in mice) *B. nodoterga* extracts were non-toxic, while *B. pernix* extract exhibit the highest toxicity among the studied species. Skin showed the highest toxic, followed by the liver. Retention time data in the LC-FLD system indicated the presence of TTX, 4-*epi*TTX, 4,9-anhydroTTX and TDA, SIM data confirmed the presence of these compounds and revealed other analogs such as 11-norTTX-6(S)-ol, 5-deoxyTTX, 11-deoxyTTX, 11-oxoTTX, 6-epiTTX. Two new components were also identified by mass spectrometry (348 and 330 Da). These unknown compounds have daughter ions similar to TTX, suggesting new putative TTX analogues.

Keywords: Tetrodotoxin brachycephalus

1. Introduction

Since the description of tetrodotoxin several studies revealed its wide distribution in terrestrial as well as marine animals (Yasumoto et al., 1989). Among fresh water and terrestrial animals TTX is known only in the amphibian class, its occurrences was reported for few families of newts (Yasumoto et al., 1986; Yasumoto et al., 1988; Yotsu et al. 1990; Yotsu-Yamashita and Mebs, 2001) and toads and frogs (Fuhrman et al., 1969; Kim et al., 1975; Mebs et al., 1995; Daly et al., 1994; Tanu, et al., 2000,). Pires et al. (2002) described its presence in *Brachycephalidae anurans*.

The Brachycephalidae family is composed by six frog species (Frost, 2002) endemic to the Brazilian Atlantic rain forest (Pombal et al., 1998). *B. ephippium* Spix, 1824 and *B. pernix* Pombal, 1998 are clearly aposematic presenting an attractive yellow warning colouration, although *B. pernix* is dark in flanks. In the genus *Brachycephalus, B. nodoterga* Miranda-Ribeiro, 1920 is the only one which has a cryptic green–yellow or green–gray colouration (Heyer et al., 1990).

Pires et al. (2002) described the occurrence of TTX, 4-epiTTX, 4,9-anhydroTTX, 11-norTTX-(S)-ol and tetrodonic acid (TDA) in *Brachycephalus ephippium*, a diurnal yellow frog from the Brazilian Atlantic rain forest. 11-oxoTTX, an analogue four to five times more toxic than TTX itself, was also benn found in *B. ephippium* extracts (Pires et al, 2003).

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Fig. 1. (A) *Brachycephalus ephippium*, (B) *Brachycephalus nodoterga*, (C) *Brachycephalus pernix* (Photos: *B. ephippium*, R.A.V. Morales; *B. nodoterga* e *B pernix*, *A. Sebben*). Scale bar, 1 mm.

This report describes the occurrence of tetrodotoxin and its analogues in tissue extracts of *B. ephippium, B. pernix* and *B. nodoterga* and demonstrates the presence of putative new TTX analogues.

2. Materials and methods

2.1. 1-Animals and extraction

Adult specimens (Fig. 1) of *B. ephippium, B. nodoterga and B. pernix* and were collected in the Brazilian Atlantic rain forest (Table 1). The animals were killed by freezing.

Whole bodies and for some animals skin and liver were combined and extracted with methanol: acetic acid (96:4, v:v) solution and stored at 4 °C for 24 h. The methanolic extracts were evaporated to dryness at 40 °C, and the residues were resuspended in deionized water.

2.2. Toxicity assay

The lethal potency of the extracts was estimated by intraperitoneal (i.p.) injection into mice (19–21 g body weight). The lethal potency was calculated from Kawabata's table for dose–death time relationship for TTX (Kawabata, 1978).

2.3. Reference toxins

Standards of TTX, 6-*epi*TTX, 4-*epi*TTX, and 4,9-anhydroTTX were prepared from the puffer fish *Sphoeroides spengleri* according to Goto et al. (1965). Tetrodonic acid was prepared according to Mosher (1986), and commercially available TTX (Sigma Chemical Company, Inc.) was used as standard compound. The identity of each reference toxin had been confirmed by mass spectrometry—MALDI-TOF (Pires et al., 2002).

2.4. Purification and analysis of the extracts

The extracts were loaded onto an ion-exchange column (Amberlite GC-50 1×3 cm –NH4+form), followed by a treatment with active charcoal (Goto et al., 1965). The semi-purified extracts were submitted to a post-column LC-FLD (Yotsu et al., 1989) with some modifications, as described by Pires et al. (2002) and to LC/MS (Tsuruda et al., 2002). For LC-MS and LC-MS/MS analysis the HPLC system was coupled to a Quattro II mass spectrometer using Z-spray ion source (Waters-Micromass ESI/MS) coupled to Class VP-10AD liquid chromatography (Shimadzu Co.).

LC-MS conditions were similar to those used in LC-FLD. However, ammonium heptafluorobutyrate of

Table 1
Distribution of toxicity and quantification of TTX-equivalents in *Brachycephalus* species (ND, not detect)

	Locality/State	Number of specimens.	Total weight (g)	Mouse bioassay (μg/g)	LC-FLD (μg/g)
B. nodoterga	Salesópolis-São Paulo	6	1.00	ND	0.76
B. ephippium	Atibaia-São Paulo	6	2.32	44.88	9.43
B. ephippium	Mogi das Cruzes-São Paulo	25	8.90	24.20	0.80
B. ephippium	Teresópolis-Rio de Janeiro	7	1.83	17.16	5.36
Skin		33	2.07	57.20	37.44
Liver		33	0.56	38.94	20.89
Ovaries		33	0.17	ND	11.70
B. pernix	Quatro Barras-Paraná	15	2.47	75.90	30.14
Skin		11	0.44	215.82	99.42
Liver		11	0.14	145.86	97.65

the mobile phase was reduced to 30 mM (pH 5.0), because high concentrations of this reagent suppress significantly the intensity of the sample signal. Flow rate was optimized to 0.5 ml/min.

Some specific parameters of the device were followed to analysis: desolvatation temperature of 350 °C, block temperature 120 °C, and cone voltage 50 V, extraction cone voltage 4 kV, capillary voltage 3.46 kV. Nitrogen was used as the inert gas of solvatation and nebulization under operational pressure of 95 psi and flow rate of 150 l/h. The mass spectrometer was operated with the MassLynxTM NT system.

The analyses were accomplished in a triple play mode: (1) ions acquisition along time in the range 100–400 Da (TIC mode—total ion current; (2) search for specific ions (SIM mode—selective ion mode) for *m/z* 290, 302, 304, 320, 336 Da corresponding to the ions (M+H+) of 11-norTTX(S)-ol; 4,9-anhydroTTX; 5-deoxyTTX and 11-deoxyTTX; TDA, 6-epiTTX, 4-epiTTX and TTX; and 11-oxoTTX, and (3) fragmentation of selected mass prior to compound identification using argon as inert gas and collision energy of 30 eV.

3. Results

In mouse bioassay, *B. pernix* proved to be the most toxic species with 75.90 μg TTX-equivalent per gram (whole body) and 215.82 μg TTX-equivalent per gram (skin) respectively, while *B. nodoterga* seemed to be non-toxic (Table 1). *B. ephippium* showed some variation in toxicity; the highest levels were found in the animals from Atibaia-SP (44.88 μg TTX-equivalent per gram), intermediate from Mogi das Cruzes-SP (24.20 μg TTX-equivalent per gram) and lowest from Teresópolis—RJ (17.16 μg TTX-equivalent per gram).

In LC-FLD analysis, the identification of tetrodotoxin, tetrodonic acid, 4,9-anhydrotetrodotoxin and 4-*epi*tetrodotoxin was achieved by retention time comparisons with those of the standard compounds (Fig. 2A).

In extracts from *B. nodoterga* only traces of TTX and 11-oxoTTX were found (Fig. 2C), although tetrodotoxin was the second major peak in extract from *B. pernix* (Fig. 2D), explaining the results obtained in mouse assay (Table 1).

The ions at *m/z* 320, 336, 302, 290 and 304 corresponding to the $M+H^+$ ions of TTX, 4-*epi*TXX, 6-*epi*TTX, 11-oxoTTX, 4,9 anhydroTTX, 11-norTTX(S)-ol, 11-deoxyTTX and 5-deoxyTTX, respectively, (Fig. 3A–E) were detected in the selected ion monitoring (SIM) mode in LC/MS. Mass analysis in SIM mode revealed the presence of two unknown components at *m/z* 348 (Fig. 3F) corresponding to peaks 9 and 11, and *m/z* 330 (Fig. 3E) corresponding to peak 10 and 14 suggesting the occurrence of four putative new analogues. A careful analysis demonstrated its ability to conversion e.g. from compound 9–11 and 10–14 suggesting a chemical isomerization process (data not shown).

The relation of the ions *m/z* 320, 348 and 330 was achieved by their fragmentation pattern and the formation of characteristic daughter ions e.g. TTX (*m/z* 320.4, 302.2, 284.2, 256.8, 214.8, 161.9, 117.1 and 99.0—Fig. 4A); *m/z* 348 Da (*m/z* 347.7, 330.2, 312.4, 293.1, 274.9, 256.7, 215.0, 196.6, 178.1, 162.6, 117.1, 99.1—Fig. 3B) and *m/z* 330 (*m/z* 329.9, 292.9, 274.9, 256.8, 214.7, 162.8 and 98.8—Fig. 4C). The presence of ion fragments at *m/z* 162 (2-aminohydroquinazoline) and *m/z* 178 (2-aminodihydroxyquinazoline) strongly suggests that these analogues are related to tetrodotoxin (Shoji et al., 2001).

4. Discussion

The aposematically coloured frogs *Brachycephalus pernix* and *B. ephippium* exhibit toxicity comparable to some *Atelopus* (Kim et al., 1975) and *Polypedates* sp. (Tanu et al., 2001). On the other hand, the cryptically coloured *B. nodoterga* was found to be non-toxic. In its natural habitat *B. nodoterga* remained motionless in the leaf litter. This strategy combined with cryptic coloration may be effective against predators.

Dendrobatids frogs are probably best known for the bright coloration (Duellman and Trueb, 1986) and extreme toxicity that characterizes the genus *Dendrobates* and especially *Phyllobates* (Daly et al., 1987). Probably this bright coloration assumes a warning advertisement of toxicity and/or unpalatability for potencials predators, unfortunately, the relation of coloration and toxicity in amphibian has rarely been examined (Summers and Clough, 2001; Duellman and Trueb, 1986). This pattern of bright coloration and toxicity seems to occurs also in TTX bearing anurans, since the aposematic *Brachycephalus* and *Atelopus* species are more toxic than cryptic species, e.g. *Colosthetus inguinalis* and *B. nodoterga.*

The biogenesis of tetrodotoxin (TTX) is still unclear. In marine animals the best-supported hypothesis is that tetrodotoxin is produced by symbiontic bacteria (Noguchi et al., 1986, Yasumoto et al., 1987; Yasumoto and Yotsu-Yamashita, 1996), but its origin in terrestrial animals remains a mystery. Up to now no TTX producing bacteria have been isolated from any amphibian species which possess TTX. The toxicity variation observed among the populations of *B. ephippium* studied, may supports an exogenous origin of this toxin.

Amphibians are important sources of TTX analogues. Chiriquitoxin, which has a –CH(OH)CH(NH2)COOH group in C11 position of the TTX molecule (Yotsu et al.,1990) was discovered in *Atelopus chiriquiensis* (Kim et al., 1975). 6-*epi*TTX was first reported in *Taricha torosa* (Yasumoto et al., 1988). Kotaki and Shimizu, 1993 observed a *N*-hydroxy- and ring deoxy-TTX analogue in *Taricha granulosa*. In this report, mass analysis indicates four

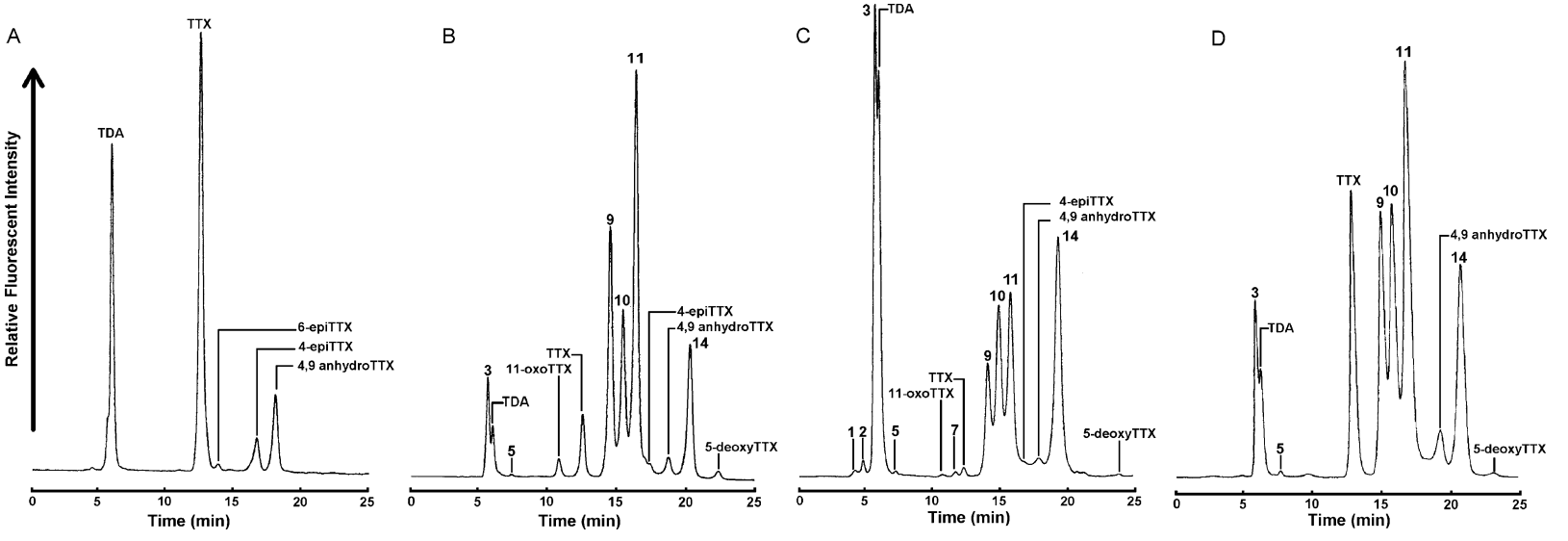

Fig. 2. Chromatogram profile of (A) standard toxins, (B) extract of *B. ephippium* from Atibaia-SP, (C) extract of *B. nodoterga*, (D) Extract of *B. pernix*. Peaks: (TDA) tetrodonic acid, (TTX) tetrodotoxin, (4-epiTTX) 4-epitetrodotoxin and (AnhydroTTX) 4,9-anhydrotetrodotoxin. HPLC analysis conditions: RP column Shimpack CLC-ODS 4.6×250 mm, mobile phase 60 mM ammonium heptafluorobutyrate in 1 mM ammonium acetate buffer (pH 5.0), flow 0.6 ml/min. Post column reaction with 4.0 N NaOH in stainless coil 0.5 mm×2.5 m at 120 °C.

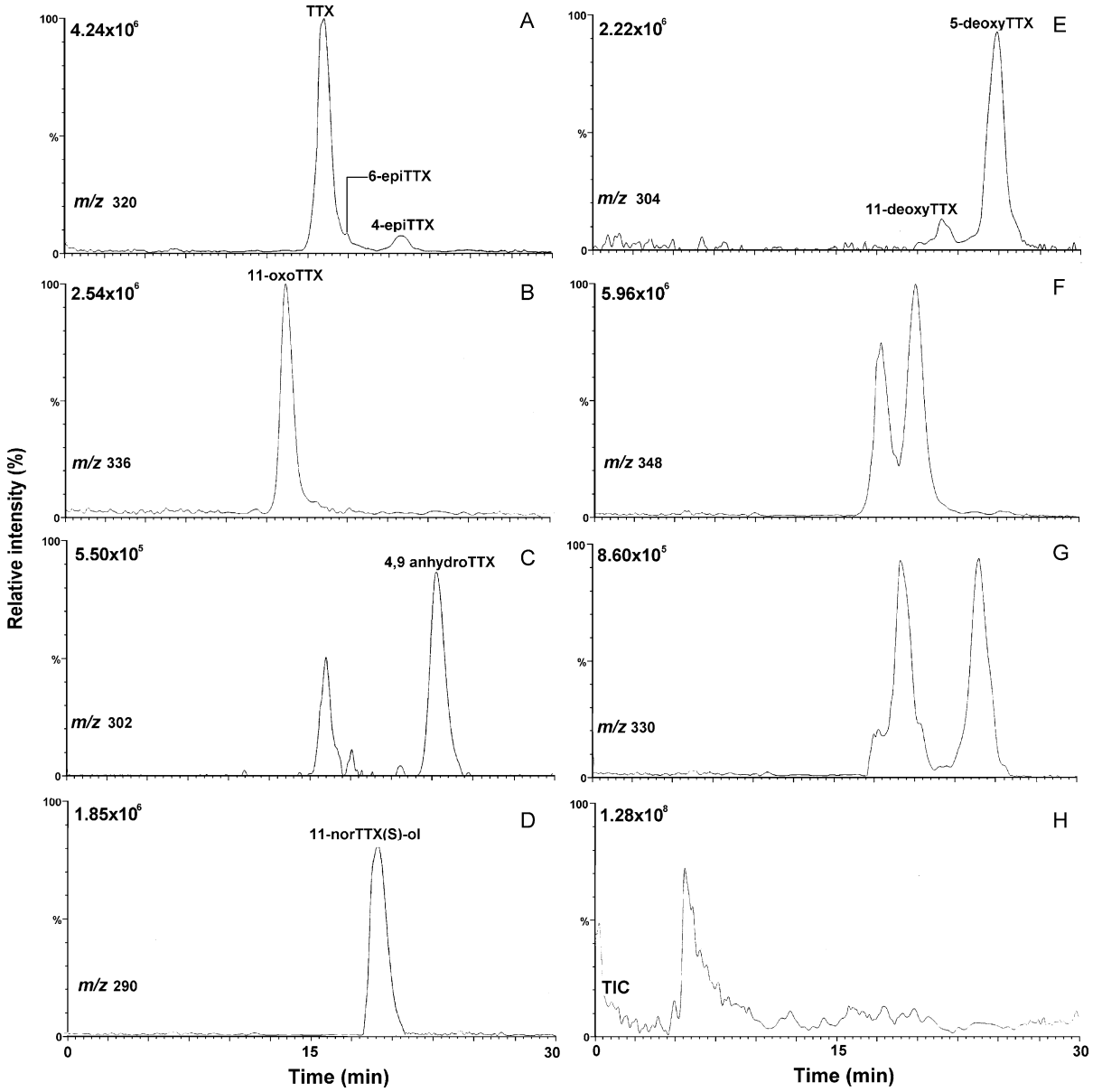

Fig. 3. LC-MS Mass spectrogram in SIM mode, exhibiting mass *m/z* (A) 320, (B) 336, (C) 302, (D) 290, (E) 304,(F) 348, (G) 330 and (H) TIC—total ionic current, assigned to TTX, 6-epiTTX e 4-epiTTX; 11-oxoTTX; 4,9-anhydroTTX, 11-norTTX-(S)-ol; 11-deoxyTTX and 5-deoxyTTX; and unknown analogues $(M+H)^+$, respectively, from *B. ephippium* extract.

unknown analogues, with additional of 28 mass units in tetrodotoxin structure, as observed in the ion *m/z* 348, suggesting the presence of an ethyl or carbonyl in the TTX molecule. The presence of two peaks at 348 *m/z* suggests isomery, while 330 *m/z* may be the anhydrous form of those isomers. The structure of these compounds is under investigation.

The identification of TTXs is limited to the LC-FLD detection system, due to C9-base conversion of different analogues. LC-MS/MS prove to be a powerful tool due to its capability to determine guanidine compounds previously undetected. Since fluorescence intensity of the analogues depends on molecular structures, the fluorescent intensity of 5-deoxyTTX and 11-deoxyTTX is approximately 1/20 and 1/100 lower than TTX, respectively. On the other hand 6-*epi*TTX and 11-norTTX-6(R)-ol is approximately 20-fold and 10-fold higher than TTX (Shoji et al., 2001).

The fragment ion spectrogram of *m/z* 348 obtained by LC/MS/MS provides a characteristic ion fragmentation pattern similar to TTX. The spectrogram also exhibited

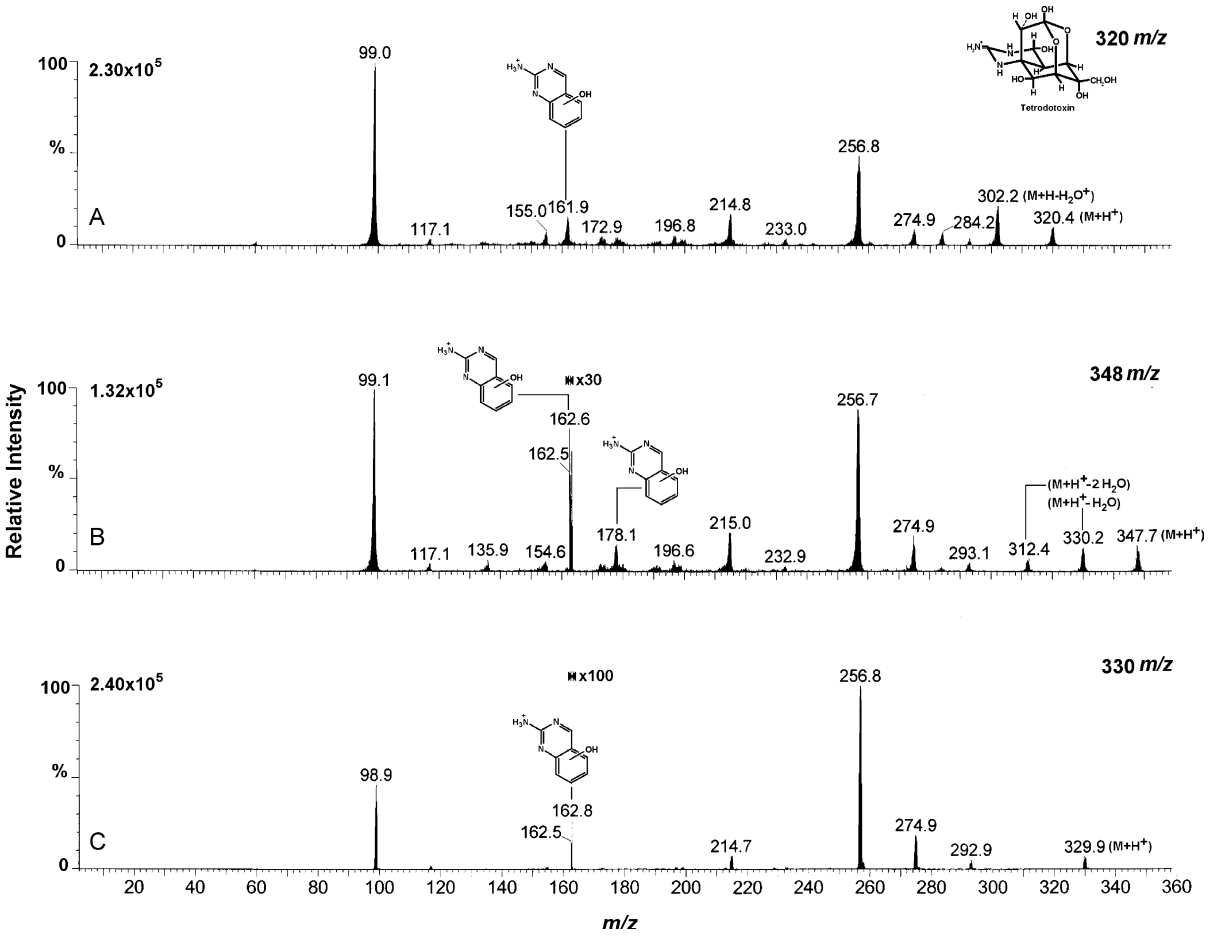

Fig. 4. Fragmentation ion profile in LC-MS/MS system of (A) TTX, (B) Peak 11 (ion *m/z* 348), and (C) Peak 14 (ion *m/z* 330).

specific fragment ions *m/z* 330 and 318 due to elimination of one and two water molecules. Ion fragments at *m/z* 162 and 178 can be interpreted as 2-aminohydroquinazoline and 2-aminodihydroxyquinazoline, respectively. These structures probably are due to bond cleavage between C8a and C9, and between C6 and C11, as suggested by Shoji et al. (2001). The fragment ion *m/z* 256 is probably generated by the loss of a 28 mass fragment from the $(MH–2H_2O)^+$ by elimination of CO- at C10 resulting from the cleavage of the bonds between C9- and C10-, C10- and C5-O- and C10- and C7-O- (Shoji et al., 2001). These data confirm that the ions *m/z* 348 and 330 belong to tetrodotoxin class by retaining of the same internal structure and the same fragmentation pattern.

Since the first detection of TTX in terrestrial vertebrates such as in the Californian newt Taricha torosa, the number of amphibian species containing TTX increased. Til now from over 5.000 species of amphibians in 44 recognized families (Frost, 2002), only six seen to contain have TTX (Daly, 2004), however TTX may be not be limited to these few families of amphibians.

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